ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2007, p. 1142–1149 Vol. 51, No. 4
0066-4804/07/$08.00+0 doi:10.1128/AAC.01227-06

Zidovudine Inhibits Thymidine Phosphorylation in the Isolated Perfused Rat Heart[▽]

Delia Susan-Resiga,† Alice T. Bentley, Matthew D. Lynx, Darcy D. LaClair, and Edward E. McKee*

Indiana University School of Medicine—South Bend, South Bend, Indiana 46617, and the University of Notre Dame, Notre Dame, Indiana 46556

Received 29 September 2006/Returned for modification 28 November 2006/Accepted 4 January 2007

Zidovudine (AZT; 3′-azido-3′-deoxythymidine), a thymidine analog, has been a staple of highly active antiretroviral therapy. It is phosphorylated in the host to the triphosphate and functions by inhibiting the viral reverse transcriptase. However, long-term use of AZT is linked to various tissue toxicities, including cardiomyopathy. These toxicities are associated with mitochondrial DNA depletion, which is hypothesized to be caused by AZT triphosphate inhibition of mitochondrial DNA polymerase γ. In previous work with isolated heart mitochondria, we demonstrated that AZT phosphorylation beyond the monophosphate was not detected and that AZT itself was a potent inhibitor of thymidine phosphorylation. This suggests an alternative hypothesis in which depletion of the TTP pool may limit mitochondrial DNA replication. The present work extends these studies to the whole cell by investigating the metabolism of thymidine and AZT in the intact isolated perfused rat heart. [^{3}H]thymidine is converted to [^{3}H]TTP in a time- and concentration-dependent manner. The level of [^{3}H]TMP is low, suggesting that the reaction catalyzed by thymidine kinase is the rate-limiting step in phosphorylation. [^{3}H]AZT is converted in a time- and concentration-dependent manner to AZT monophosphate, the only phosphorylated product detected after 3 h of perfusion. Both compounds display negative cooperativity, similar to the observations with cloned and purified mitochondrial thymidine kinase 2. The presence of AZT in the perfusate inhibits the phosphorylation of [^{3}H]thymidine with a 50% inhibitory concentration of 24 ± 4 μM. These data support the hypothesis that AZT-induced mitochondrial cardiotoxicity may be caused by a limiting pool of TTP that lowers mitochondrial DNA replication.

Zidovudine (AZT; 3′-azido-3′-deoxythymidine), a thymidine analog, is a member of the class of compounds called nucleoside analog reverse transcriptase inhibitors (NRTIs). Members of this class of drugs are phosphorylated by host cell enzymes to their triphosphates, which inhibit the human immunodeficiency virus (HIV) reverse transcriptase and/or act as chain terminators when they are incorporated into viral DNA, since these analogs cannot form 3′-phosphodiester bonds. AZT was the first drug approved by the Food and Drug Administration for the treatment of AIDS. In early monotherapy regimens, AZT was given alone at a dose of 1,200 mg per day (3). After some months of therapy, AZT was associated with a variety of common tissue toxicities that included myopathy, dilated cardiomyopathy, hepatotoxicity, various cytopenias, and lactic acidosis that were serious enough to result in the discontinuation of therapy in about 25 to 30% of patients (1, 5, 7, 9–11, 27, 29, 34, 37). In affected tissues, the mitochondria were abnormal, with reduced cristae and mitochondrial DNA (mtDNA) depletion (18, 19, 22). In present AIDS therapy, referred to as highly active antiretroviral therapy (HAART), AZT remains in common use but is given at a lower dose (600 mg per day) and in combination with a second NRTI and a viral protease inhibitor. As a result, the cardiovascular toxicity of the AZT used in HAART is now a rare event, and the main toxicities include lipodystrophy and cytopenias (2, 12, 28). AZT monotherapy is currently used to prevent the transmission of HIV from mothers to their babies prior to delivery and during the baby's first postnatal month (33). AZT is also a mainstay of HIV treatment in many resource-limited settings around the world (36). Understanding the mechanism of toxicity of AZT is of critical importance both in designing therapy to limit toxicity and in the rational design of less toxic therapeutic compounds.

The phosphorylation of AZT is mediated by cellular enzymes of the thymidine salvage pathway, which convert thymidine to TTP. This pathway has two routes. In replicating cells, the major route is cytosolic, in which the initial phosphorylation of thymidine to TMP is catalyzed by thymidine kinase 1 (TK1), and the second phosphate is added by thymidylate kinase to yield TDP. TDP can be phosphorylated by several different cytosolic diphosphokinases to supply TTP to the nucleus for replication and repair. TDP and TTP can be transported across the mitochondrial inner membrane by the deoxynucleotide carrier, which also supplies TTP for mitochondrial DNA replication. However, the expression of TK1 is closely tied to the cell cycle and has negligible activity in cells not in S phase (8). The second route of thymidine salvage is accomplished by transport of the deoxynucleoside into the mitochondrial matrix and phosphorylation by thymidine kinase 2 (TK2) to TMP. In previous work, we showed that isolated rat heart and liver mitochondria are unable to transport TMP and that the second phosphorylation is also carried out in the matrix by a previously uncharacterized matrix thymidylate kinase activity to yield TDP (20, 24). The TDP can be phosphorylated by a matrix diphos-

* Corresponding author. Mailing address: Indiana University School of Medicine—South Bend, South Bend, IN 46617. Phone: (574) 631-7193. Fax: (574) 631-7821. E-mail: McKee.6@nd.edu.

† Present address: Departments of Biochemistry and Medicine, McGill University, and The Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada H3G 1A4.

▽ Published ahead of print on 12 January 2007.

phokinase to TTP, which either can be used in mitochondrial replication or can be transported out to be used in the nucleus for DNA repair.

The cytosolic route of AZT phosphorylation in growing cells with active TK1 has been well studied. AZT is readily converted to AZT 5′-monophosphate (AZT-MP). However, AZT-MP is a poor substrate for the cytosolic thymidylate kinase, resulting in the accumulation of AZT-MP and a relatively low level of formation of AZT 5′-triphosphate (AZT-TP) (15). We have recently characterized the mitochondrial route of AZT phosphorylation in isolated mitochondria from rat heart and liver and have shown that AZT is phosphorylated to AZT-MP by TK2, but at a significantly slower rate than is thymidine (20, 24). As with TMP, AZT-MP does not appear to be transported across the inner mitochondrial membrane (24). We were unable to detect either AZT 5′-diphosphate (AZT-DP) or AZT-TP in these labeling studies, suggesting that AZT-MP is also a poor substrate for the mitochondrial thymidylate kinase activity. Finally, the rate of dephosphorylation of AZT-MP in the matrix by the mitochondrial 5′-nucleotidase is only about 4% of that of TMP (23), perhaps allowing AZT-MP to accumulate in the mitochondria.

The mechanism for the mitochondrial toxicity of NRTIs is not completely understood. The prevailing hypothesis is that the NRTI triphosphates block mtDNA replication by inhibition of the mtDNA polymerase γ or by incorporation into mtDNA, where they act as chain terminators (17). However, while there is a good correlation between the toxicity of other NRTIs and the ability of NRTI triphosphates to inhibit polymerase γ, this does not appear to be true for AZT-TP, which has a 50% inhibitory concentration (IC_{50}) of >100 μM, a value much greater than those of the other NRTIs (16, 22). Furthermore, as noted above, the level of AZT-TP observed even in replicating tissues with an active TK1 is several orders of magnitude below 100 μM. In nonreplicating tissues, such as the adult heart, where much of the toxicity is observed, the level of AZT-TP has not been determined and may be even lower. We have developed an alternative mechanism for AZT toxicity from the results of studies with mitochondria isolated from rat heart and liver. In both tissues, AZT is a competitive inhibitor of thymidine phosphorylation, with K_i values of 10.6 ± 4.5 μM AZT for heart mitochondria and 14.0 ± 2.5 μM AZT for liver mitochondria (21). Additionally, the IC_{50}s with 1 μM thymidine, the approximate physiological concentration (38), were determined to be 7.0 ± 1.0 μM AZT for heart mitochondria and 14.4 ± 2.6 μM AZT for liver mitochondria (20, 24). These values are for inhibition by AZT itself, not by AZT-TP, and are relatively close to the steady-state 1.5-h-postdosing peak serum concentration (2.32 μM; range, 0.19 to 5.46 μM) of patients given 250 mg of AZT orally every 4 h. This dosage is slightly higher than the standard dosage of 200 mg every 4 h for AZT monotherapy seen in the late 1980s and early 1990s but is still in the area of dose-independent kinetics (3). In adult nonreplicating tissues such as the heart, TK1 and enzymes of the deoxynucleotide de novo synthesis pathway are probably not expressed. Thus, the synthesis of TTP in these cells may depend solely on the mitochondrial TK2 pathway. We hypothesize that through inhibition of thymidine phosphorylation by TK2, AZT may cause a depletion of the TTP pool, which in turn could result in the inhibition of mtDNA replication and cause mtDNA depletion (35).

The work presented here extends our understanding of thymidine and AZT metabolism beyond the mitochondrion to include the whole cell of a nonreplicating tissue. The heart perfusion system should provide an accurate representation of rat heart thymidine and AZT metabolism in vivo. The data show that [^{3}H]thymidine was readily phosphorylated to TTP in the perfused heart, while [^{3}H]AZT was phosphorylated only to the monophosphate. The presence of AZT in the perfusate was a strong inhibitor of thymidine phosphorylation. The results support our hypothesis that cardiac toxicity is not caused by AZT-TP inhibition of the mitochondrial DNA polymerase but, rather, is caused by the inhibited thymidine phosphorylation potentially disrupting the TTP pool.

MATERIALS AND METHODS

Rat heart perfusion. Adult outbred Sprague-Dawley rats (weight, 250 to 400 g) were obtained from Harlan (Indianapolis, IN). The rats were injected intraperitoneally with heparin in saline (0.4 U/g) 10 to 30 min before perfusion and were subsequently anesthetized with sodium pentobarbital (100 mg/kg of body weight intraperitoneally). When they were fully anesthetized, rats were incised laterally below the diaphragm, the chest cavity was opened, and the heart was rapidly removed and placed in cold saline solution. The hearts were immediately placed on a Langendorff perfusion apparatus and perfused at 37°C by a modified Langendorff procedure, exactly as described previously (25). Briefly, isolated rat hearts were subjected to an initial 10 min of nonrecirculating perfusion with Krebs-Henseleit bicarbonate buffer gassed with 95% O_2–5% CO_2 and containing 15 mM glucose and the plasma levels of all amino acids. Next, the hearts were perfused at 60 mm Hg for a fixed time (5 min to 180 min) with 30 ml of recirculating perfusion buffer. The recirculating perfusion buffer contained 0.1% bovine serum albumin, insulin (2.5 mU/ml), and [^{3}H]deoxynucleoside (thymidine or AZT), in addition to the components described above. The levels of labeled thymidine or AZT and the total concentration (unlabeled and labeled) of deoxynucleoside used are outlined below.

Preparation of perfused heart and perfusate for HPLC analysis. While being perfused, the hearts were frozen in tongs cooled in liquid nitrogen. From each frozen wafer of heart, 0.1- to 0.3-g pieces were individually homogenized in 2 ml of 5% trichloroacetic acid, followed by precipitation on ice (10 min of incubation) and a 3-min centrifugation at 2,000 × *g* at 4°C. A measured volume of the resultant supernatant (heart extract) was neutralized by addition of AG-11A8 resin (0.5-g/ml extract) and filtered, and the labeled deoxynucleoside/deoxynucleotide pools were analyzed by high-pressure liquid chromatography (HPLC), as described in the section "HPLC analysis of thymidine, AZT, and phosphorylated nucleotides." The isolated perfused heart method does not allow the isolation of heart mitochondria; therefore, the results obtained reflect pathways pertaining to the whole tissue. In control experiments, the rates of recovery of [^{3}H]TTP and [^{3}H]thymidine added to the unlabeled perfused heart samples and subjected to this extraction procedure were close to 100%. The breakdown of TTP to TDP was less than 2%.

A 0.5-ml sample was removed from each initial and final recirculating perfusion buffer (perfusate) for HPLC analysis. Each sample was precipitated with an equal volume of 10% trichloroacetic acid, after which the same procedure used for the heart samples was followed.

Phosphorylation of thymidine and AZT in isolated perfused rat heart. For the experiments on the time course of phosphorylation, isolated rat hearts were perfused for time intervals ranging from 5 min to 120 min in the presence of 1 μM perfusate [^{3}H]thymidine or [^{3}H]AZT (2,200 dpm/pmol). At the end of the interval the hearts were freeze-clamped and extracted as described above. In the kinetics of phosphorylation experiments, the isolated rat hearts were perfused for 30 min with various concentrations of the labeled precursor: [^{3}H]thymidine at 0.1 μM to 50 μM (2,200 to 300 dpm/pmol) or [^{3}H]AZT at 0.1 μM to 20 μM (4,440 to 555 dpm/pmol). Each heart was freeze-clamped and extracted as described above.

The effect of AZT on the conversion of [^{3}H]thymidine to its phosphorylated forms in the isolated perfused heart was studied by perfusing rat hearts for a fixed time period (30 min) in the presence of [^{3}H]thymidine (1 μM; 2,200 dpm/pmol) under conditions in which the levels of unlabeled AZT were varied from 0 to 100

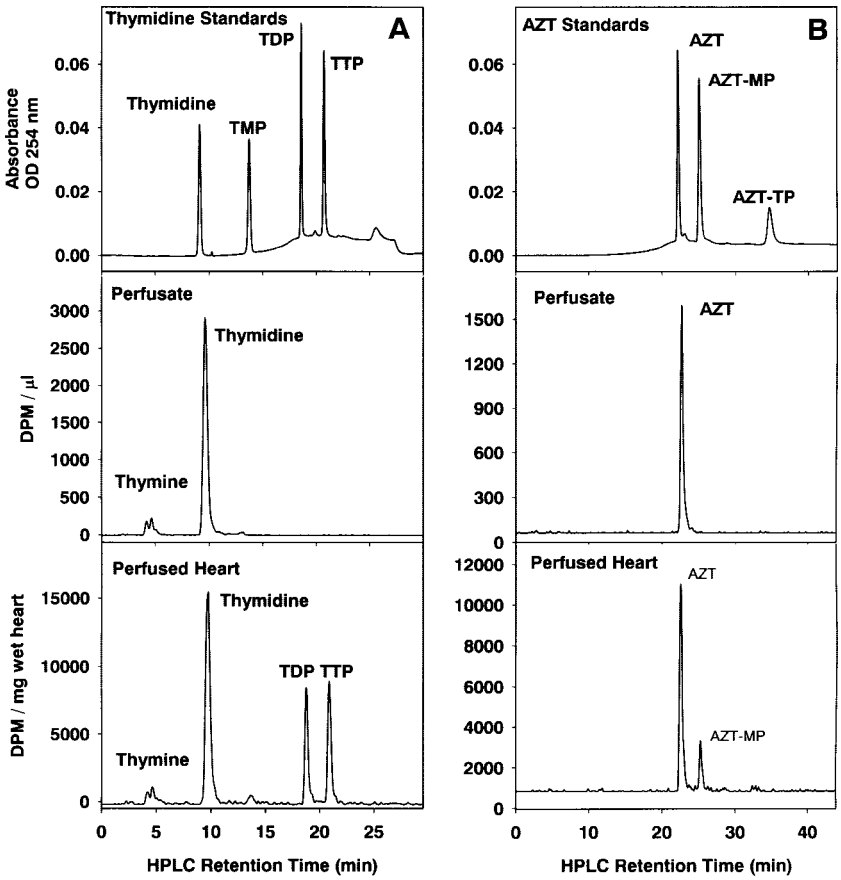

FIG. 1. Representative HPLC separation profiles of thymidine and AZT nucleosides and nucleotides in final perfusate and isolated perfused rat heart. Isolated rat hearts were perfused for 30 min in the presence of [^{3}H]thymidine (2,200 dpm/pmol; 1 μM) (A) or [^{3}H]AZT (2,200 dpm/pmol; 1 μM) (B). Hearts and perfusate samples were prepared as described in Materials and Methods. (Top panels) Thymidine standards (A) and AZT standards (B) detected by absorption at 254 nm; (middle panels) final perfusate activity (dpm/μl) detected by an in-line scintillation counter; (bottom panels) perfused heart activity (dpm/mg [wet weight] of heart) detected with an in-line scintillation counter. OD, optical density.

μM. For each set of experiments, the heart extracts were prepared and analyzed as outlined in the previous section.

HPLC analysis of thymidine, AZT, and phosphorylated nucleotides. The peaks corresponding to [^{3}H]thymine, [^{3}H]thymidine, [^{3}H]AZT, and their phosphorylated forms were identified and quantitated from samples prepared as described above ("Preparation of perfused heart for HPLC analysis") by reverse-phase HPLC over an Alltech nucleotide/nucleoside column connected in-line with a UV detector (254 nm) and a flowthrough scintillation counter, as described previously (24). As a reference, at the beginning of each HPLC analysis, a set of thymidine standards or AZT standards was separated and detected by measurement of the absorption at 254 nm.

Data treatment. The values of the reported parameters (V_{max}, K_m, and IC_{50}) and their respective standard deviations were obtained by best-fit graphical analysis of the experimental data by using Sigma Plot 9.0 software. Data obtained as dpm per mg (wet weight) of heart were divided by the specific radioactivity of the labeled precursor and are presented as pmol per mg (wet weight) of heart.

RESULTS

Thymidine and AZT phosphorylation in isolated perfused rat heart. The goal of this study was to quantitate the uptake and conversion of the labeled compound (thymidine or AZT) to phosphorylated intermediates in the whole heart. Hence, it was important that the energy charge of the system (the nucleotide pools) be maintained. To accomplish this, at specified perfusion times, the hearts were freeze-clamped with tongs cooled in liquid nitrogen. Typical phosphorylation profiles in isolated rat hearts perfused with [^{3}H]thymidine or [^{3}H]AZT for 120 min are shown in Fig. 1. The results clearly indicate that [^{3}H]thymidine was converted by the isolated perfused rat heart to labeled TMP, TDP, and TTP, whereas [^{3}H]AZT was detected only as labeled AZT-MP. AZT-DP and AZT-TP remained undetected even after 3 h of perfusion (data not shown). We have readily extracted and detected both AZT-DP and AZT-TP by this extraction procedure in other systems (data not shown); thus, the failure to detect these forms was not due to losses during the extraction procedure. Becher et al. have reported that AZT-TP may be converted to the NRTI stavudine (d4T-TP) in patients taking AZT (4). There is some controversy as to whether or not this conversion occurs (6, 26). Nonetheless, our chromatograms (Fig. 1) showed no detectable levels of d4T, which had a much earlier retention time than AZT, suggesting that this conversion, if it occurred in the perfused heart, was below the limits of detection in our system. In other work we demonstrated that d4T was not phosphorylated and had no effect on thymidine phosphorylation in the perfused rat heart (unpublished data). A small amount of thymidine, but not AZT, was degraded to thymine and related breakdown products (10.6% ± 1.2% over 2 h). These break-

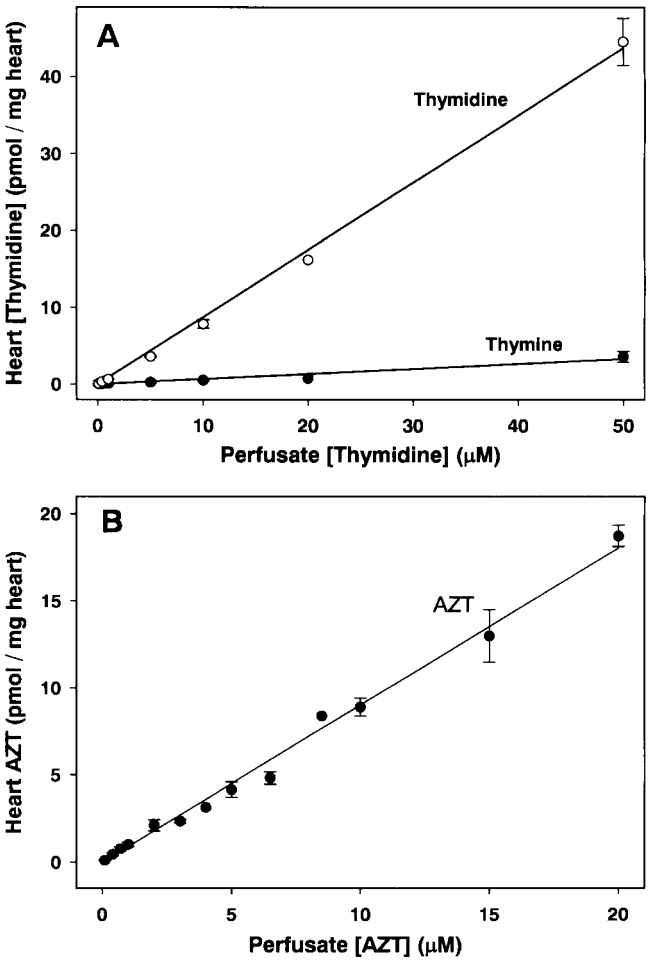

FIG. 2. Uptake of thymidine and AZT into isolated perfused rat heart. Isolated rat hearts were perfused for 30 min in the presence of various concentrations of [^{3}H]thymidine (0.1 μM to 50 μM; specific radioactivity, 2,200 to 300 dpm/pmol) (A) or in the presence of various concentrations of [^{3}H]AZT (0.1 μM to 20 μM; specific radioactivity, 4,440 to 555 dpm/pmol) (B). The perfused hearts were freeze-clamped and prepared for HPLC analysis, as described in Materials and Methods. The amount of [^{3}H]thymidine (A) or [^{3}H]AZT (B) in the heart was quantitated by HPLC and in-line scintillation counting. The results are presented as picomoles of labeled thymidine, thymine, or AZT per milligram (wet weight) of heart divided by the specific radioactivity of the thymidine or AZT pool. The plotted data represent the means ± standard errors of the means of three (A) or three to seven (B) experiments. See the text for additional details.

down products, observed at a retention time of ~4 min in both the heart and the final perfusate, were not observed in perfusates that recirculated for 2 h in the absence of a heart (data not shown), demonstrating that this breakdown was catalyzed by heart enzymes, presumably thymidine phosphorylase. AZT was not a good substrate for this enzyme, and degradation was not detected. Phosphorylated intermediates of thymidine and AZT were never observed in the perfusate. The phosphorylated intermediates observed for both thymidine and AZT in the whole heart were identical to the intermediates seen in isolated heart mitochondria (24).

Uptake of thymidine and AZT into the heart and degradation to thymine. The amount of [^{3}H]thymidine (Fig. 2A) and [^{3}H]AZT (Fig. 2B) observed in the heart after 30 min of perfusion displayed a linear dependence on the concentration of labeled nucleoside in the perfusate. The slope of the line for thymidine was 0.87, and that for AZT was 0.90. Since 1 g (wet weight) of heart was about 85% water, these data suggested a passive transport mechanism in which the concentration of nucleoside in intracellular water was the same as that in the perfusate. As noted above, a small amount of thymidine is degraded to thymine, which averaged 3 to 4% of the total label in the heart at all concentrations of perfusate thymidine (Fig. 2A). AZT concentrations remained quite stable over 30 min of perfusion, and the breakdown to thymine was negligible (Fig. 2B).

Time course of thymidine and AZT phosphorylation in isolated perfused rat heart. The purpose of the time course experiments was to determine the ability of the intact rat heart to take up and phosphorylate labeled thymidine or its analog, AZT. The formation of the phosphorylated form(s) of the labeled precursor was measured as a function of time. The rate of appearance of the phosphorylated forms of thymidine and AZT in the isolated perfused heart was measured with 1 μM [^{3}H]thymidine (Fig. 3A) or 1 μM [^{3}H]AZT (Fig. 3B). Total thymidine phosphorylation proceeded in a linear fashion, reaching a plateau after 60 min. TTP was the major phosphorylated form at all time points, followed by TDP. The levels of TMP were negligible until 2 h of perfusion, when the levels rose somewhat, indicating that the phosphorylation of thymidine to TMP is the rate-limiting step. The rate of appearance of AZT-MP was linear for up to 30 min of perfusion, when it reached a plateau. The plateau value for AZT-MP was only 25% of the value reached for total thymidine phosphorylation. Other phosphorylated forms of AZT were not detectable even after 3 h of perfusion.

Kinetics of thymidine and AZT phosphorylation in isolated perfused rat heart. The kinetics of thymidine phosphorylation were determined by using concentrations of thymidine between 0.1 and 50 μM perfused through the heart for 30 min. A standard Michaelis-Menten plot is shown in Fig. 4A. The apparent V_{max} calculated from total thymidine phosphorylation, defined as the sum of TMP, TDP, and TTP, was 3.1 ± 0.5 pmol per mg (wet weight) of heart per 30 min. The apparent K_m for thymidine phosphorylation was 15.7 ± 6.0 μM. Others have previously shown that thymidine and AZT phosphorylation by purified recombinant TK2 displayed an unusual negative cooperativity (43) that was also observed by our laboratory for thymidine and AZT phosphorylation in isolated heart and liver mitochondria (20, 24). To detect this possibility we replotted our data as an Eadie-Hofstee plot (Fig. 4B), which was distinctly biphasic, consistent with negative cooperativity, and yielded a K_{m1} of 0.8 μM and a K_{m2} of 10.4 μM. The negative cooperativity was confirmed by a Hill plot (Fig. 4B, inset) with a slope of 0.74, which is less than unity. The kinetics of phosphorylation of AZT to AZT-MP were carried out in a manner similar to that used for the thymidine experiments with concentrations between 0.1 and 20 μM in the isolated perfused heart for 30 min. A standard Michaelis-Menten plot (Fig. 5A) yielded an apparent V_{max} of 0.71 ± 0.45 pmol per mg (wet weight) of heart per 30 min. The apparent K_m for AZT phosphorylation was 3.4 ± 0.6 μM. When the data were replotted in the Eadie-Hofstee format, two distinct slopes were also observed for AZT phosphorylation, which corresponded to

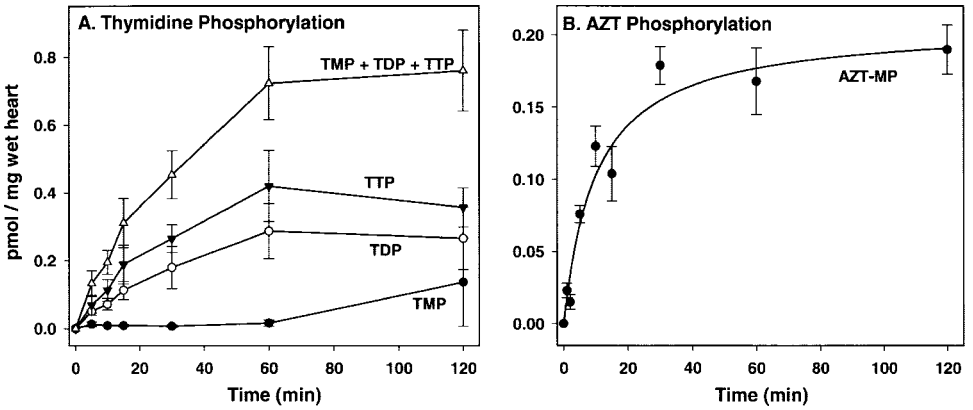

FIG. 3. Time course of phosphorylation of thymidine and AZT in isolated perfused rat heart. Isolated rat hearts were perfused for various fixed time periods in the presence of [^{3}H]thymidine (2,200 dpm/pmol; 1 μM) (A) or in the presence of [^{3}H]AZT (2,200 dpm/pmol; 1 μM) (B). The results were obtained as described in the Fig. 2 legend. The plotted data represent the means ± standard errors of the means for three hearts at each time point.

K_{m1} of 1.0 μM and K_{m2} of 4.2 μM. The Hill plot yielded a coefficient of n of 0.65. Thus, the phosphorylation of AZT is also consistent with negative cooperativity and suggests that the enzyme responsible for phosphorylation in the perfused heart is the mitochondrial TK2.

AZT inhibition of [^{3}H]thymidine phosphorylation in isolated perfused rat heart. Previous work demonstrated that AZT is a potent inhibitor of thymidine phosphorylation in mitochondria isolated from the heart and liver (20, 21, 24). To determine if AZT had a similar effect on thymidine phosphorylation in intact whole tissue, the hearts were perfused for 30 min with 1 μM [^{3}H]thymidine (approximating physiological thymidine levels [38]) plus 0 to 200 μM AZT. As the concentration of AZT present was increased, the total phosphorylation of thymidine was decreased (Fig. 6). The IC_{50} under the conditions described above was 24.4 ± 4.0 μM. Although this value is somewhat higher than the IC_{50} values obtained with isolated mitochondria under similar conditions, the data nevertheless demonstrate for the first time in whole tissue that AZT is a potent inhibitor of thymidine phosphorylation. The nucleoside analogs d4T and lamivudine do not appear to be toxic to the rat heart and do not inhibit thymidine phosphorylation in the perfused heart (data not shown).

DISCUSSION

The results presented here demonstrate that the isolated perfused rat heart can be used as a model to study the metabolism of thymidine and thymidine analogs, such as AZT, in an intact organ system. The data observed should closely reflect the in vivo heart metabolism of these compounds. The heart is a nonreplicating tissue in which the demand for deoxynucleotides is limited to nuclear DNA repair and mtDNA replication. The mechanisms that supply cells of this type with deoxynucleoside triphosphates are largely unknown, but the salvage of deoxynucleosides represents a much more efficient pathway than de novo synthesis.

Thymidine levels in the heart mirrored the levels in the perfusate at concentrations up to 50 μM (Fig. 2A), demonstrating that transport into the cell was mediated by an equilibrative nucleoside transporter (ENT), such as the previously characterized ENT1 or ENT2 (14). Once thymidine was in the cell, it was readily converted into TTP in a time- and concentration-dependent manner (Fig. 3A). The kinetics of thymidine phosphorylation displayed unusual negative cooperativity (Fig. 4B) that had been previously observed by our group for thymidine phosphorylation in isolated heart and liver mitochondria (20, 24) and by others with purified thymidine kinase 2 (43). Thus, in the perfused heart, thymidine is transported into the mitochondrial matrix and is phosphorylated to TMP by TK2 as the rate-limiting enzyme. Earlier work with isolated heart mitochondria indicated that TMP is not transported across the inner mitochondrial membrane and is phosphorylated a second time in the matrix by a TMP kinase to TDP (24). While a cytosolic TMP kinase has been identified and well characterized, a mitochondrial TMP kinase remains unidentified.

Our present study shows that AZT, a thymidine analog, followed the same mitochondrial pathway as thymidine, except that only AZT-MP was observed as a product. This confirmed an earlier finding, in which AZT-MP was the only form detected in isolated heart and liver mitochondria (20, 24). It is well known that AZT-MP is a poor substrate for the cytosolic thymidylate kinase, causing a "bottleneck" in the activation of AZT to AZT-TP (15). The data presented here indicate that AZT-MP is an even worse substrate for the mitochondrial thymidylate kinase. Since AZT-MP, like TMP, is not transported across the inner mitochondrial membrane and is also a poor substrate for the mitochondrial 5′-nucleotidase (23), it is possible that the amount of AZT-MP could rise to significant levels within the matrix. However, this was not observed during the time frame of the present experiments.

The current prevailing hypothesis used to explain the toxicities of NRTIs suggests that NRTI triphosphates inhibit mtDNA polymerase γ, causing an inhibition of mtDNA replication and resulting in the observed depletion of mtDNA in the affected tissues. There is good agreement between the toxicities of most of these drugs and the degree to which they are predicted to inhibit mtDNA replication, with the major exception being AZT (16). AZT-TP is, at best, only a weak

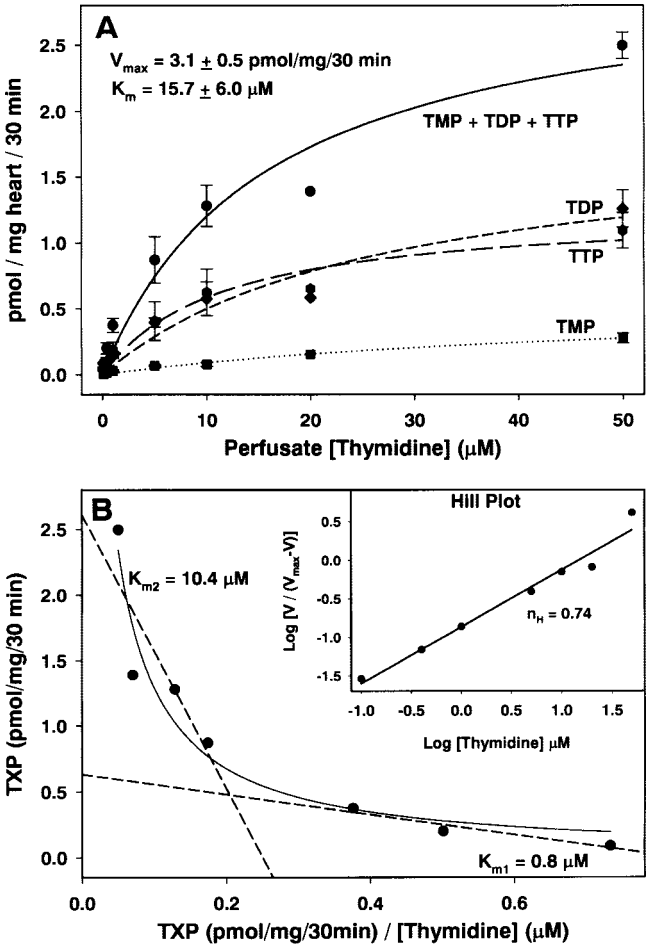

FIG. 4. Kinetics of thymidine phosphorylation in isolated perfused rat heart. Isolated hearts were perfused with [^{3}H]thymidine as described in the Fig. 2 legend. (A) The rate of thymidine phosphorylation was monitored by measuring the amounts of TMP, TDP, and TTP formed over 30 min of perfusion and was expressed as pmol of product per mg (wet weight) of heart per 30 min. The plotted data represent the means ± standard errors of the means of three experiments. The data for the sum of TMP, TDP, and TTP phosphorylation were fitted to the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$, where v is velocity (pmol/mg heart/30 min of product TMP, TDP, TTP, or their sums) and $[S]$ is the thymidine concentration, yielding an apparent V_{max} of 3.1 ± 0.5 pmol per mg (wet weight) of heart per 30 min and an apparent K_m of 15.7 ± 6.0 μM. (B) The Michaelis-Menten equation was rearranged to a linear form by use of the Eadie-Hofstee equation: $v = -K_m v/[S] + V_{max}$. In this rearrangement, the data can be approximated with two straight lines with slopes corresponding to K_{m1} of 0.8 μM and K_{m2} of 10.4 μM. The biphasic nature of this plot suggests negative cooperativity for the kinetics of thymidine phosphorylation in isolated heart. The curve through the data is not a fit and is drawn to emphasize the nonlinear nature of the results. (Inset) The negative cooperativity for total thymidine phosphorylation (TXP [the sum of TMP, TDP, and TTP]) is further supported by a Hill plot slope (n_H) < 1.

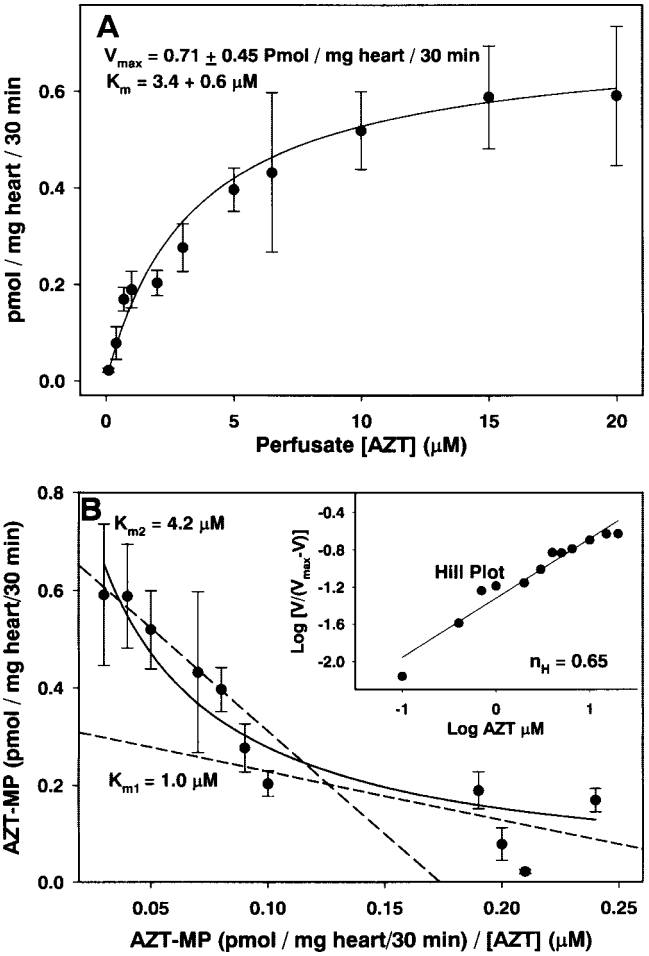

FIG. 5. Kinetics of AZT phosphorylation in isolated perfused rat heart. Isolated hearts were perfused with [^{3}H]AZT as described in the Fig. 2 legend. (A) The rate of AZT phosphorylation was determined by measuring the amount of AZT-MP formed over 30 min of perfusion and is expressed as pmol per mg (wet weight) of heart per 30 min. The plotted data represent the means ± standard errors of the means for three to seven hearts. The data were fitted to the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$, where v is velocity (pmol/mg heart/30 min of product AZT-MP) and $[S]$ is the AZT concentration, yielding an apparent V_{max} of 0.71 ± 0.4 pmol per mg (wet weight) of heart per 30 min and an apparent K_m of 3.4 ± 0.6 μM. (B) Eadie-Hofstee plot, as defined in the legend to Fig. 4. The data were approximated with two straight lines with slopes corresponding to K_{m1} of 1.0 μM and K_{m2} of 4.2 μM. As indicated in the legend to Fig. 4, the biphasic nature of this plot suggests negative cooperativity for the kinetics of AZT phosphorylation in the isolated heart. The curve through the data is not a fit and is drawn to emphasize the nonlinear nature of the results. (Inset) The negative cooperativity for AZT phosphorylation is further supported by a Hill plot slope (n_H) < 1.

inhibitor of polymerase γ (IC_{50}, >100 μM). From the results of in vitro studies with polymerase γ alone, the inhibitory effect of AZT-TP is unlikely to be associated with the level of tissue toxicity observed (16, 22). Furthermore, as noted by others, the synthesis of AZT-TP is quite limited in replicating cells, and in the perfused heart study presented here there was no evidence of AZT-TP synthesis. AZT-MP has been shown to inhibit the exonuclease activity of polymerase γ, but at a concentration of 1 to 2 mM (19). The V_{max} value of 0.71 pmol per mg (wet weight) of heart obtained in Fig. 5 for the production of AZT-MP and a value of 50 mg of mitochondria per g (wet weight) of heart (13) yields a value of AZT-MP of 0.0142 pmol per mg mitochondrial protein. If this is all contained within the 1-μl/mg protein matrix space, the concentration of AZT-MP would equal 14.2 μM, which is nearly 100-fold below the inhibitory concentration of 1 to 2 mM. In the present study we

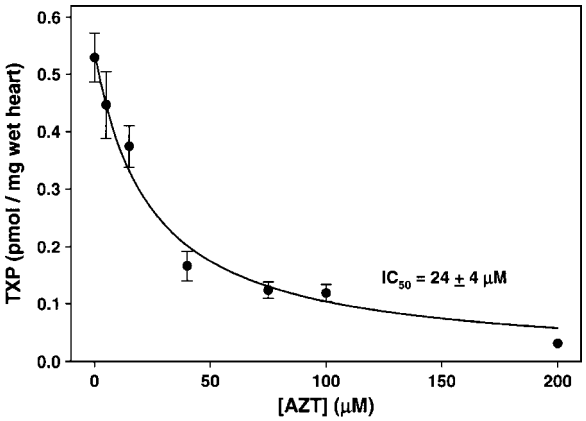

FIG. 6. AZT inhibition of thymidine phosphorylation in isolated perfused rat heart. Isolated rat hearts were perfused for 30 min in the presence of [^{3}H]thymidine (2,200 dpm/pmol; 1 μM) under conditions in which the levels of unlabeled AZT were varied from 0 to 200 μM. The results were calculated for the sum of formation of TMP, TDP, and TTP, as described in the Fig. 3 legend. The results represent the means ± standard errors of the means for three to five hearts at each concentration of AZT. The curve through the data represents the fit to the equation $v = (V_{max}\,IC_{50})/([I]\,IC_{50})$, where v is pmol/mg heart of product TXP generated in 30 min and $[I]$ is the AZT concentration, which yields an IC_{50} of 24 ± 4 μM. TXP, sum of TMP, TDP, and TTP.

demonstrate that the prodrug AZT inhibits thymidine phosphorylation with an IC_{50} of 24.4 ± 4.0 μM, and in isolated heart mitochondria we obtained a lower IC_{50} of 7.0 ± 1.0 μM (20, 21, 24). While the IC_{50} value in perfused heart is well above the concentration range of AZT in serum (3), it is much closer to physiological relevance than the concentrations of AZT-MP and AZT-TP required for toxicity, as discussed above. Furthermore, since we have demonstrated that AZT inhibition of thymidine phosphorylation is competitive (21), the IC_{50} value reflects in part the thymidine concentration. The dose-response curve presented here was done with a thymidine perfusate concentration of 1 μM, to approximate a physiological concentration. However, serum levels of thymidine vary considerably within and between mammalian species, with values as low as 0.2 μM having been reported (38), which would significantly lower the IC_{50} value reported here. In addition, the inhibition observed here was from a single acute dose of AZT in an otherwise healthy heart. While the effects of cumulative doses of AZT, together with the presence of HIV, on AZT inhibition of TK2 are not yet known, cumulative treatment could potentiate TK2 inhibition. Based on the findings obtained with isolated mitochondria (20, 21, 24), we proposed an alternative mechanism to account for the observed AZT-linked mtDNA depletion in nonreplicating tissues, such as muscle and heart. In the heart, and perhaps most nonreplicating tissues, TTP appears to be synthesized solely via the mitochondrial TK2 pathway. These tissues do not express TK1 (8), nor is there any evidence for the expression of thymidylate synthase, which converts dUMP to TMP (G. Morris and E. E. McKee, unpublished data). Thus, inhibition of TK2 by the prodrug AZT is likely to decrease mtDNA, both by limiting the amount of TTP for mtDNA replication and by creating unbalanced deoxynucleotide pools. Imbalances in any of the deoxynucleotide pools are known to cause mtDNA depletion (30, 35). Further support for this hypothesis comes from mutations characterized in humans that cause a partial deficiency in TK2. Individuals with this disorder have a severe myopathy that is lethal in childhood and that is associated with muscle mtDNA depletion (31, 32). Too little is known to be able to speculate on the time frame required to observe a decrease in the TTP pool. However, work is in progress to determine if the inhibition of TK2 in the perfused rat heart model leads to a decrease in the TTP pool that can be measured in the relatively short duration of a heart perfusion experiment (<3 h).

In previous work, AZT inhibition of thymidine phosphorylation in isolated mitochondria was shown to be competitive (21). Thus, if the toxicity of AZT is a function of inhibiting TK2 and decreasing the TTP pool, then increasing thymidine levels in the patient may provide a possible route to overcoming this toxicity. While thymidine has not been directly used, similar work in this area has been done by the use of supplementation with uridine. Walker et al. have demonstrated that the addition of uridine to HepG2 and 3T3-F442A cells exposed to several NRTIs, including AZT, helped to lessen the apparent toxicity in vitro (40, 42). Additionally, the effect of uridine on the amelioration of the toxicity of NRTIs in humans is presently in clinical trials of mitocnol, a sugar cane extract containing a high concentration of uridine, as an oral supplement (39, 41). The mechanism of action for uridine in the systems described above is not clear, but it may follow a pathway in which the uridine is salvaged and converted to TTP, supplementing the intracellular pool.

It should be noted that the results presented here were obtained with healthy adult rat hearts. The effects of prolonged AZT treatment on the heart with regard to potential changes in gene expression that could alter thymidine or AZT metabolism are not known and could alter the interpretation of these data. Understanding these possible effects will require studying thymidine and AZT metabolism in heart from rats that have been treated with AZT for a prolonged period.

ACKNOWLEDGMENTS

This work was supported by grant R01HL72710 from the National Institutes of Health to E. E. McKee and a predoctoral fellowship from the American Heart Association Midwest Affiliate to M. D. Lynx.

REFERENCES

1. **Arnaudo, E., M. Dalakas, S. Shanske, C. T. Moraes, S. DiMauro, and E. A. Schon.** 1991. Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. Lancet **337:**508–510.
2. **Barile, M., D. Valenti, E. Quagliariello, and S. Passarella.** 1998. Mitochondria as cell targets of AZT (zidovudine). Gen. Pharmacol. **31:**531–538.
3. **Barnhart, E. R.** 1990. Physicians' desk reference, p. 799–801. Medical Economics Company, Oradell, NJ.
4. **Becher, F., A. G. Pruvost, D. D. Schlemmer, C. A. Creminon, C. M. Goujard, J. F. Delfraissy, H. C. Benech, and J. J. Grassi.** 2003. Significant levels of intracellular stavudine triphosphate are found in HIV-infected zidovudine-treated patients. AIDS **17:**555–561.
5. **Benbrik, E., P. Chariot, S. Bonavaud, M. Ammi-Said, E. Frisdal, C. Rey, R. Gherardi, and G. Barlovatz-Meimon.** 1997. Cellular and mitochondrial toxicity of zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) on cultured human muscle cells. J. Neurol. Sci. **149:**19–25.
6. **Benech, H., F. Becher, A. Pruvost, and J. J. Grassi.** 2006. Is stavudine triphosphate a natural metabolite of zidovudine? Antimicrob. Agents Chemother. **50:**2899.
7. **Chariot, P., I. Drogou, I. de Lacroix-Szmania, M. C. Eliezer-Vanerot, B. Chazaud, A. Lombes, A. Schaeffer, and E. S. Zafrani.** 1999. Zidovudine-induced mitochondrial disorder with massive liver steatosis, myopathy, lactic acidosis, and mitochondrial DNA depletion. J. Hepatol. **30:**156–160.
8. **Coppock, D. L., and A. B. Pardee.** 1985. Regulation of thymidine kinase activity in the cell cycle by a labile protein. J. Cell. Physiol. **124:**269–274.

9. **Corcuera, T., M. J. Alonso, A. Picazo, F. Gomez, M. Roldan, M. Abad, E. Munoz, and A. Lopez-Bravo.** 1996. Hepatic morphological alterations induced by zidovudine (ZDV) in an experimental model. Pathol. Res. Pract. **192:**182–187.
10. **Cupler, E. J., M. J. Danon, C. Jay, K. Hench, M. Ropka, and M. C. Dalakas.** 1995. Early features of zidovudine-associated myopathy: histopathological findings and clinical correlations. Acta Neuropathol. (Berlin) **90:**1–6.
11. **Dalakas, M. C., I. Illa, G. H. Pezeshkpour, J. P. Laukaitis, B. Cohen, and J. L. Griffin.** 1990. Mitochondrial myopathy caused by long-term zidovudine therapy. N. Engl. J. Med. **322:**1098–1105.
12. **Deveaud, C., B. Beauvoit, S. Hagry, A. Galinier, A. Carriere, B. Salin, J. Schaeffer, S. Caspar-Bauguil, Y. Fernandez, J. B. Gordien, D. Breilh, L. Penicaud, L. Casteilla, and M. Rigoulet.** 2005. Site specific alterations of adipose tissue mitochondria in 3′-azido-3′-deoxythymidine (AZT)-treated rats: an early stage in lipodystrophy? Biochem. Pharmacol. **70:**90–101.
13. **Idell-Wenger, J. A., L. W. Grotyohann, and J. R. Neely.** 1982. An improved method for isolation of mitochondria in high yields from normal, ischemic, and autolyzed rat hearts. Anal. Biochem. **125:**269–276.
14. **Lai, Y., C. M. Tse, and J. D. Unadkat.** 2004. Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. J. Biol. Chem. **279:**4490–4497.
15. **Lavie, A., I. Schlichting, I. R. Vetter, M. Konrad, J. Reinstein, and R. S. Goody.** 1997. The bottleneck in AZT activation. Nat. Med. **3:**922–924.
16. **Lee, H., J. Hanes, and K. A. Johnson.** 2003. Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. Biochemistry **42:**14711–14719.
17. **Lewis, W., and M. C. Dalakas.** 1995. Mitochondrial toxicity of antiviral drugs. Nat. Med. **1:**417–422.
18. **Lewis, W., B. Gonzalez, A. Chomyn, and T. Papoian.** 1992. Zidovudine induces molecular, biochemical, and ultrastructural changes in rat skeletal muscle mitochondria. J. Clin. Investig. **89:**1354–1360.
19. **Lim, S. E., and W. C. Copeland.** 2001. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase gamma. J. Biol. Chem. **276:**23616–23623.
20. **Lynx, M. D., A. T. Bentley, and E. E. McKee.** 2006. 3′-Azido-3′-deoxythymidine (AZT) inhibits thymidine phosphorylation in isolated rat liver mitochondria: a possible mechanism of AZT hepatotoxicity. Biochem. Pharmacol. **71:**1342–1348.
21. **Lynx, M. D., and E. E. McKee.** 2006. 3′-Azido-3′-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Biochem. Pharmacol. **72:**239–243.
22. **Martin, J. L., C. E. Brown, N. Matthews-Davis, and J. E. Reardon.** 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. **38:**2743–2749.
23. **Mazzon, C., C. Rampazzo, M. C. Scaini, L. Gallinaro, A. Karlsson, C. Meier, J. Balzarini, P. Reichard, and V. Bianchi.** 2003. Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy. Biochem. Pharmacol. **66:**471–479.
24. **McKee, E. E., A. T. Bentley, M. Hatch, J. Gingerich, and D. Susan-Resiga.** 2004. Phosphorylation of thymidine and AZT in heart mitochondria: elucidation of a novel mechanism of AZT cardiotoxicity. Cardiovasc. Toxicol. **4:**155–167.
25. **McKee, E. E., B. L. Grier, G. S. Thompson, A. C. Leung, and J. D. McCourt.** 1990. Coupling of mitochondrial metabolism and protein synthesis in heart mitochondria. Am. J. Physiol. **258:**E503–E510.
26. **Melendez, M., R. Blanco, W. Delgado, R. Garcia, J. Santana, H. Garcia, O. Rosario, and J. F. Rodriguez.** 2006. Lack of evidence for in vivo transformation of zidovudine triphosphate to stavudine triphosphate in human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. **50:**835–840.
27. **Mhiri, C., M. Baudrimont, G. Bonne, C. Geny, F. Degoul, C. Marsac, E. Roullet, and R. Gherardi.** 1991. Zidovudine myopathy: a distinctive disorder associated with mitochondrial dysfunction. Ann. Neurol. **29:**606–614.
28. **Moh, R., C. Danel, S. Sorho, D. Sauvageot, A. Anzian, A. Minga, O. B. Gomis, C. Konga, A. Inwoley, D. Gabillard, E. Bissagnene, R. Salamon, and X. Anglaret.** 2005. Haematological changes in adults receiving a zidovudine-containing HAART regimen in combination with cotrimoxazole in Cote d'Ivoire. Antivir. Ther. **10:**615–624.
29. **Olano, J. P., M. J. Borucki, J. W. Wen, and A. K. Haque.** 1995. Massive hepatic steatosis and lactic acidosis in a patient with AIDS who was receiving zidovudine. Clin. Infect. Dis. **21:**973–976.
30. **Pontarin, G., P. Ferraro, M. L. Valentino, M. Hirano, P. Reichard, and V. Bianchi.** 2006. Mitochondrial DNA depletion and thymidine phosphate pool dynamics in a cellular model of mitochondrial neurogastrointestinal encephalomyopathy. J. Biol. Chem. **281:**22720–22728.
31. **Saada, A., A. Shaag, and O. Elpeleg.** 2003. mtDNA depletion myopathy: elucidation of the tissue specificity in the mitochondrial thymidine kinase (TK2) deficiency. Mol. Genet. Metab. **79:**1–5.
32. **Saada, A., A. Shaag, H. Mandel, Y. Nevo, S. Eriksson, and O. Elpeleg.** 2001. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat. Genet. **29:**342–344.
33. **Sia, J., S. Paul, R. M. Martin, and H. Cross.** 2004. HIV infection and zidovudine use in childbearing women. Pediatrics **114:**e707–e712.
34. **Sinnwell, T. M., K. Sivakumar, S. Soueidan, C. Jay, J. A. Frank, A. C. McLaughlin, and M. C. Dalakas.** 1995. Metabolic abnormalities in skeletal muscle of patients receiving zidovudine therapy observed by ^{31}P in vivo magnetic resonance spectroscopy. J. Clin. Investig. **96:**126–131.
35. **Song, S., Z. F. Pursell, W. C. Copeland, M. J. Longley, T. A. Kunkel, and C. K. Mathews.** 2005. DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. Proc. Natl. Acad. Sci. USA **102:**4990–4995.
36. **Stringer, J. S., I. Zulu, J. Levy, E. M. Stringer, A. Mwango, B. H. Chi, V. Mtonga, S. Reid, R. A. Cantrell, M. Bulterys, M. S. Saag, R. G. Marlink, A. Mwinga, T. V. Ellerbrock, and M. Sinkala.** 2006. Rapid scale-up of antiretroviral therapy at primary care sites in Zambia: feasibility and early outcomes. JAMA **296:**782–793.
37. **Tanuma, J., A. Ishizaki, H. Gatanaga, Y. Kikuchi, S. Kimura, M. Hiroe, and S. Oka.** 2003. Dilated cardiomyopathy in an adult human immunodeficiency virus type 1-positive patient treated with a zidovudine-containing antiretroviral regimen. Clin. Infect. Dis. **37:**e109–e111.
38. **Traut, T. W.** 1994. Physiological concentrations of purines and pyrimidines. Mol. Cell. Biochem. **140:**1–22.
39. **Venhoff, N., M. Zilly, D. Lebrecht, D. Schirmer, H. Klinker, J. Thoden, P. Langmann, and U. A. Walker.** 2005. Uridine pharmacokinetics of mitocnol, a sugar cane extract. AIDS **19:**739–740.
40. **Walker, U. A., M. Auclair, D. Lebrecht, M. Kornprobst, J. Capeau, and M. Caron.** 2006. Uridine abrogates the adverse effects of antiretroviral pyrimidine analogues on adipose cell functions. Antivir. Ther. **11:**25–34.
41. **Walker, U. A., and N. Venhoff.** 2005. Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity. Antivir. Ther. **10**(Suppl. 2)**:**M117–M123.
42. **Walker, U. A., N. Venhoff, E. C. Koch, M. Olschewski, J. Schneider, and B. Setzer.** 2003. Uridine abrogates mitochondrial toxicity related to nucleoside analogue reverse transcriptase inhibitors in HepG2 cells. Antivir. Ther. **8:**463–470.
43. **Wang, L., B. Munch-Petersen, A. Herrstrom Sjoberg, U. Hellman, T. Bergman, H. Jornvall, and S. Eriksson.** 1999. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett. **443:**170–174.